Biochimica et Biophysica Acta xxx (2013) xxx–xxx

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Review

Methionine oxidation and reduction in proteins☆,☆☆

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ARTICLE INFO

Article history:
Received 5 February 2013
Received in revised form 24 April 2013
Accepted 27 April 2013
Available online xxxx

Keywords:
Methionine
Methionine sulfoxide
Methionine sulfoxide reductase
Oxidant defense

ABSTRACT

Background: Cysteine and methionine are the two sulfur containing amino acids in proteins. While the roles of protein-bound cysteinyl residues as endogenous antioxidants are well appreciated, those of methionine remain largely unexplored.
Scope: We summarize the key roles of methionine residues in proteins.
Major conclusion: Recent studies establish that cysteine and methionine have remarkably similar functions.
General significance: Both cysteine and methionine serve as important cellular antioxidants, stabilize the structure of proteins, and can act as regulatory switches through reversible oxidation and reduction. This article is part of a Special Issue entitled Current methods to study reactive oxygen species — pros and cons.

1. Introduction

Methionine and cysteine are the two sulfur-containing amino acids that are present in peptides and proteins. It would not be difficult for most of us to list the functions of cysteine residues in proteins. Well-known roles include antioxidant defense, catalysis, protein structure, and redox sensing and regulation [1]. In contrast, we might have difficulty listing the functions of methionine (Met) residues, other than its well-known role in protein initiation. Biochemistry texts typically treat Met as a generic hydrophobic amino acid, readily interchangeable with other residues such as leucine or valine. This concept is woefully outdated. Over the last 15 years, research from a number of laboratories supports the concept that Met in proteins shares much of the same job description as cysteine, playing important roles in oxidant defense redox sensing and regulation as well as protein structure.

The most important common characteristic of cysteine and Met residues in proteins is that both are subject to reversible oxidation and reduction, mediated either enzymatically or non-enzymatically. While cysteine forms cystine through a disulfide linkage, Met forms methionine sulfoxide (MetO) by addition of oxygen to its sulfur atom. Disulfides may be reduced back to the thiol form by various reductases, often utilizing thioredoxin [2]. MetO is reduced back to Met by the methionine sulfoxide reductases, thioredoxin-dependent enzymes that are virtually universal among aerobic organisms [3,4]. Oxidation of Met to MetO introduces a chiral center at the sulfur atom so there are two epimers of MetO; R-MetO and S-MetO. While an epimerase could theoretically exist that interconverts the forms, none has been found so far. Instead, organisms have two types of methionine sulfoxide reductases (Msr). MsrA specifically reduces S-MetO, but not R-MetO. Conversely, MsrB reduces R-MetO, but not S-MetO. The existence of MsrA has been appreciated for decades, while the existence of MsrB was only reported recently [5]. To date, there is substantial experimental evidence to support the importance of MsrA, both *in vivo* and *in vitro*. Knocking out MsrA caused increased susceptibility to oxidative stress in mice [6], yeast [7], and bacteria [8–10]. Conversely, overexpressing MsrA conferred increased resistance to oxidative stress in *Drosophila* [11], *Saccharomyces* [12], *Arabidopsis* [13], PC-12 cells [14], and human T cells [12]. Interestingly, overexpression in *Drosophila* doubled the lifespan of the flies [11]. Critical functions for MsrB remain to be defined given its more recent discovery.

While cysteine is well-recognized for the ease of its oxidation, it is often not appreciated that Met can be readily oxidized to MetO [15,16]. Indeed, the standard redox potential for the two electron reduction of dimethyl sulfoxide is +160 mV [17] while that for cystine is +220 mV [18]. Cysteine is easily oxidized when ionized to its thiolate, but is difficult to oxidize when in the thiol form [19]. Cysteine residues at the active sites of enzymes such as phosphatases, dehydrogenases, reductases, and peroxidases generally have a low pK_a which makes them readily oxidizable [19]. However, the majority of cysteine residues, including those in glutathione, have a pK_a around 8.3–8.7 and are not easily oxidized at physiological pH, unless the oxidation is catalyzed by an enzyme. In contrast, oxidation of Met residues is

Abbreviations: A_2M, $\alpha_2$macroglobulin; MetO, methionine sulfoxide; Msr, methionine sulfoxide reductase

☆ This article is part of a Special Issue entitled Current methods to study reactive oxygen species — pros and cons.

☆☆ Funding: This study was supported by the Intramural Research Program of the National Heart, Lung, and Blood Institute.

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http://dx.doi.org/10.1016/j.bbagen.2013.04.038

essentially independent of pH [20]. *In vitro*, hypochlorous acid (HOCl), a major halogenated oxidant generated by leukocytes, reacts rapidly with Met at physiological pH [20,21], but hydrogen peroxide does not, although the rate can be accelerated by the bicarbonate/carbon dioxide present *in vivo* [22]. The relative importance *in vivo* of cysteine and Met as antioxidants has not been established and most likely varies depending on the oxidizing agent.

2. Methionine residues in proteins as antioxidants

2.1. $\alpha_2$macroglobulin

$\alpha_2$macroglobulin (A_2M) is a high molecular weight (~725 kDa), physiologically important plasma proteinase inhibitor that targets a wide variety of proteinases [23,24]. Acting in a "venus-flytrap"-like mode that serves to entrap proteinases in a molecular cage [25], A_2M normally circulates as a homotetrameric molecule that is disulfide linked into a pair of dimers that are held in association by strong non-covalent forces. In its open conformation, target proteinases cleave an exposed "bait" region within the A_2M tetramer that triggers the structural changes that result in the irreversible entrapment of the protease. Often acting at sites of inflammation where reactive oxygen and nitrogen species are at relatively high concentration, it was initially thought that A_2M was resistant to oxidative modification [26]. However, studies by Weiss and colleagues demonstrated that the antiproteinase was sensitive to oxidative modification by activated neutrophils, HOCl or derivative chloramines (a natural byproduct of neutrophil-generated HOCl following its reaction with amines) [24–26]. In the course of these reactions, Met residues in A_2M readily react with chlorinated oxidants, consuming the reactive species while oxidizing Met to its corresponding sulfoxide [26]. These oxidations had previously not been observed because only the activity of A_2M was monitored, and, at least initially, the oxidation of Met residues proceeds without loss of anti-proteinase activity [26]. Our detailed study of the oxidation reaction established that each subunit of A_2M consumes 8 mol of chloramine without any loss of anti-proteinase function [26]. During a second phase of oxidative modification, the A_2M is inactivated with loss of activity proceeding in a manner directly proportional to the consumption of chlorinated oxidants. At this point, each subunit had consumed 16 mol of chloramine, but only 14 Met residues were oxidized. Further studies demonstrated that a single tryptophan residue in each subunit was being oxidized by the remaining chloramine and that the decrease in total tryptophan residues (from 11 to 10) was directly proportional to loss of anti-proteinase activity in tandem with the dissociation of the tetrameric A_2M into dimers [26].

That the tryptophan became susceptible to oxidation only after conversion of more than 8 Met residues to their sulfoxides could be explained by perturbation of the normal A_2M structure by the presence of the additional sulfoxides, leading to an inactive conformation with incidental exposure of the normally buried tryptophan. However, the ability of A_2M to tolerate oxidation of 8 Met per subunit without loss of activity led to the proposal of an alternative hypothesis in which these residues functioned as antioxidants that protected the critical tryptophan residue from oxidation. Using high pressure liquid chromatography and mass spectrometry, we have determined that the oxidized Trp residue is Trp413. We then generated recombinant wild-type and Trp413Ala and Trp413Phe site-specific mutants in order to test whether loss of Trp413 was sufficient to cause dissociation of the tetramer to the dimer. Native gel electrophoresis demonstrated that wild-type A_2M is, as expected, a tetramer, but both Trp413 mutants are almost completely dissociated to the dimer (Fig. 1). Thus, Trp413 must be intact for normal subunit–subunit interaction. We therefore proposed that the purpose of the multiple, readily oxidized Met residues in A_2M was to act as a last line of defense against reactive species that had evaded low molecular weight antioxidants and enzymatic antioxidant systems [26–28]. In other words, Met residues serve as innate antioxidants or "molecular bodyguards", positioned to intercept reactive species to prevent damage to other residues critical to the function of the protein.

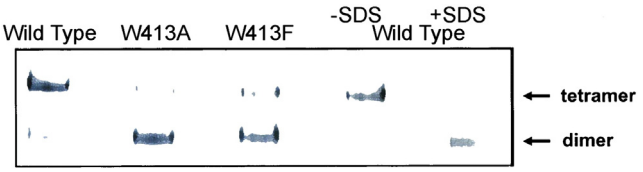

Fig. 1. Native gel electrophoresis of wild-type and Trp413 mutant A_2M. Samples were analyzed on a single native gel. The wild-type sample in the far right lane was made 2% in SDS to dissociate tetramers to disulfide-linked dimers. The proteins were produced as follows: Chinese hamster ovary CHO-K1 cells were purchased from ATCC (catalog CCL-61, Manassas, Virginia, USA) and grown in DMEM (Gibco-BRL 10566-016, Life Technologies, Grand Island, New York, USA) containing 10% fetal calf serum. Cells were maintained in 5% CO_2 and 5% oxygen. The human A_2M gene sequence encoding wild type (WT), Trp413Ala, Trp413Phe was cloned into pcDNA 3.1 (+) (Invitrogen V790-20, Life Technologies, Grand Island, New York, USA). CHO-K1 cells were stably transfected with the expression vectors. The stably transfected cells were selected in DMEM with 10% FBS containing 3 mg/ml Geneticin (Invitrogen 10131-027). Selected cell lines were then grown in CHO serum-free culture medium (BioWhittaker 12-029Q, Walkersville, Maryland, USA) without Geneticin. After 73 h in culture, 30 ml of medium was collected and concentrated to 1.5 ml through a centrifugal filter YM10 (EMD Millipore, Billerica, Massachusetts, USA). The concentrated samples were subjected to native gel electrophoresis on a 6% Tris-glycine gel (EC6068, Invitrogen) run at 125 V for 130 min at room temperature. Proteins were electroblotted to a polyvinylidene difluoride membrane (LC2002, Invitrogen), incubated with rabbit anti-human A_2M as the primary antibody (DAKO A0033, DAKO, Carpinteria, California, USA) and an anti-rabbit IgG conjugated to alkaline phosphatase (475-1516, KPL, Gaithersburg, Maryland, USA) as the secondary antibody. A_2M was visualized by incubation with nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (50-81-08, KPL).

2.2. Glutamine synthetase

To date, there are no crystal structures for native A_2M. While there is a low resolution structure of methylamine-reacted A_2M (a form of A_2M wherein the anti-proteinase undergoes conformational changes similar to those elicited by targeted proteinases) [29], this conformation is known to be very different from that of the native A_2M [30,31], thereby complicating efforts to characterize oxidative events at the structural level. We therefore turned our focus to glutamine synthetase from *Escherichia coli*, for which several excellent crystal structures have been determined [32–34]. Exposure of the enzyme to varying concentrations of hydrogen peroxide generated a series of preparations with an increasing content of MetO; no other covalent modifications were detected [27]. Eight of the 16 Met residues could be oxidized without loss of catalytic activity. Mapping of the oxidizable Met residues revealed that all were surface exposed; conversely, the residues that remained unoxidized were buried. More detailed examination of the topographic distribution of the oxidizable Met residues was intriguing as these residues were found to line the bay leading from the surface of the enzyme to its active site (Fig. 4 in [27]). In other words, these Met residues are mustered in a phalanx guarding the active site where they too function as macromolecular bodyguards.

2.3. Enlistment of methionines as antioxidants is widespread

In addition to glutamine synthetase and A_2M, many other proteins have likely evolved with similar placement of "guardian" Met residues. For example, mammalian 15-lipoxygenases undergo an apparently irreversible auto-inactivation during the catalytic cycle. The enzyme contains ~16 Met residues, and oxidation of a single Met near the active site correlates with inactivation [35]. However, Gan and colleagues reported that site-specific replacement of the oxidizable Met590 by a leucine residue yielded an enzyme that remained sensitive to auto-inactivation [36]. Thus, while Met590 is critical to in the regulation or catalytic activity of the lipoxygenase, it presumably functions as a guardian antioxidant for the active site.

Please cite this article as: G. Kim, et al., Methionine oxidation and reduction in proteins, Biochim. Biophys. Acta (2013), http://dx.doi.org/10.1016/j.bbagen.2013.04.038